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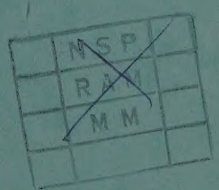

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STUDIES ON THE SURVIVAL OF PLANT PATHOGENIC BACTERIA IN STERILIZED AND UNSTERILIZED SOIL

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(Received for publication, May 1961)

Bacterial plant pathogens are known to be attacked by soil microorganisms, especially actinomycetes (Goodman and Henry, 1945; Landergin and Lochhead, 1948; Patrick, 1954; Anonymous, 1956; Mazunina, 1958; Chiu, Dih and Yuen, 1958). The present study deals with the survival of plant pathogenic bacteria in sterilized and unsterilized soil.

Materials and Methods

Xanthomonas malvacearum (E.F.Sm.) Dowson from diseased cotton leaves and *Erwinia carotovora* (Jones) Holland from soft-rot of onion bulbs were isolated by the tissue culture method.

Three hundred g. portions of black loam soil, collected from Annamalai University Experimental Farm were placed in each of the 1 lb. wide mouth bottles and sterilized at 20 lb. pressure for 3 hr. Twentyfour hour old cultures of *X. malvacearum* and *E. carotovora*, growing in nutrient broth, were diluted with sterile distilled water and inoculated into the soils. The moisture content was adjusted to 50% of the water holding capacity of soil. The bottles were incubated at room temperature (26°—28°C) and the moisture

content of the soil was maintained at the same level by adding required amount of water every day. Unsterilized soils were inoculated with the pathogens in the way described above and also kept as control. Ken Knight's agar was used for actinomycetes, yeast extract dextrose agar for bacteria and potato dextrose agar for fungi in order to estimate their numbers by plating technique. The number of organisms/g. of soil was calculated on dry weight basis.

To study the survival of bacteria when added along with their host tissues, freshly collected diseased cotton leaves and freshly harvested diseased onion bulbs were buried in pots containing sterile and unsterile soil. At periodical intervals specimens were unearthed and studied for the presence of pathogens as well as for other microbial populations. The infected host tissues were also streaked on Ken Knight's agar medium in Petri-dishes. The colonies of actinomycetes developing were isolated and screened for their antagonistic activity against bacteria, by the usual cross streak assay method. The actinomycetes were grown in 75 ml. portions of glucose asparagine medium contained in 250 ml. Erelenmeyer flasks. The flasks were shaken on a wrist action shaking machine. The culture filtrates were tested at periodical intervals, after passing through a sintered glass filter, by incorporating serial dilutions of the filtrate in nutrient agar in Petri-dishes and streaking the test organisms.

Results

X. malvacearum and *E. carotovora* survived for a longer period in sterilized than in the unsterilized soil. In sterilized soil their numbers increased slightly at first and then there was a gradual decrease. There was an increase in the actinomycetes population of the unsterilized soil by the addition of plant pathogens (Table I). These results are in agreement with Waksman and Boyd (1940). The inhibitory effect of the natural flora of seeds on seed-borne pathogens has been shown by Henry and Campbell (1938), Diachun (1939) and Stanford and Broadfoot (1931).

The survival period of *X. malvacearum* and *E. carotovora*, when added to sterile and non-sterile soil along with the infected tissues, was much less than when these bacteria were added as cell suspensions. The former species survived in sterilized soil for 62 days and in unsterilized one for 24 days and the latter for 79 and 31 days (compare this with the results given in Table I).

By examining the infected tissues of cotton leaves and onion bulbs, buried in sterile and unsterile soil, a number of strains of bacteria and actinomycetes were isolated. Of these, 29 isolates of actinomycetes obtained from the tissues imbeded in unsterile soil, were tested for their antagonistic effect against *X. malvacearum*, *X. citri*, *E. carotovora*, *Saccharomyces cerevisiae* and *Pseudomonas* sp. Most of them showed marked inhibitory effect either on one or more of the test organisms. Five showed inhibitory effect on *X. malvacearum* and six on *E. carotovora*. Of these, two which showed the highest inhibitory effect on *Xanthomonas* spp. were selected for further studies. These strains (No. X-1 and X-10) were grown in glucose-asparagine medium on shake machine and the filtrates were assayed, using *X. malvacearum*, *X. citri* and *Pseudomonas* sp. as test organisms (Table II). The maximum concentration of the inhibitory substance was produced usually on the fourth day. The presence of antagonistic microorganisms in soil, inhibiting the growth of plant pathogenic bacteria *in vitro*, has also been reported by Katznelson (1940),

TABLE I

Survival of X. malvacearum *and* E. carotovora *in soil when inoculated as cell suspensions*

Treatment	Microorganisms in millions/g. of soil on dry weight basis											
	Counts at different intervals (days)											
	8	16	37	73	110	121	131	141	153	162	171	180
Unsterile soil (control)												
Bacteria	17·32	15·22	18·91	17·88	14·61	12·63	12·61	15·76	14·13	12·44	16·13	12·01
Actinomycetes	0·65	0·82	1·11	0·67	0·98	0·71	0·64	0·91	1·24	0·59	1·06	1·34
Fungi	4·72	4·11	4·19	4·94	4·15	5·19	3·42	4·05	4·46	3·84	2·76	3·39
Total	22·69	20·15	24·21	23·49	19·74	18·53	16·67	20·72	19·83	16·87	19·95	16·74
Unsterile soil + *X. malvacearum*												
X. malvacearum	63·52	58·10	43·35	20·02	1·48	0	0	0	0	0	0	0
Other bacteria	23·52	20·12	22·54	17·47	17·29	18·38	—	—	—	—	—	—
Actinomycetes	0·82	1·04	2·12	2·28	2·32	2·55	—	—	—	—		—
Fungi	3·81	2·53	2·74	2·22	2·52	2·11	—	—	—	—	—	—
Total	91·67	81·79	70·75	41·99	23·61	23·04	—	—	—	—	—	—
Sterile soil + *X. malvacearum*	78·48	80·17	75·27	46·15	25·89	13·43	5·84	2·96	0·85	0	0	0
Unsterile soil + *E. carotovora*												
E. carotovora	75·55	78·99	50·21	31·25	9·42	2·21	0	0	0	0	0	0
Other bacteria	17·19	21·44	20·91	22·24	19·56	16·69	15·16	—	—	—	—	—
Actinomycetes	0·69	1·09	1·88	1·76	2·16	2·45	2·16	—	—	—	—	—
Fungi	3·60	3·11	4·31	4·12	3·14	3·21	2·92	—	—	—	—	—
Total	97·03	104·63	77·31	59·37	34·28	24·56	20·24	—	—	—	—	—
Sterile soil + *E. carotovora*	82·21	91·52	95·24	58·74	35·21	17·78	9·19	6·68	2·64	3·54	1·38	0

Landergin and Lochhead (1948), Rouatt, Lechevalier and Waksman (1951), Bhide and Moniz (1952), Munnecke (1956), Chiu, Dih and Yuen (1958), and Mazunina (1958).

TABLE II

Dilutions of actinomycetes culture filtrates inhibiting the growth of bacteria*

Age of the filtrate (days)	Actinomyces X-1			Actinomyces X-10		
	X. citri	*X. malva-cearum*	*Pseudomonas* sp.	*X. citri*	*X. malva-cearum*	*Pseudomonas* sp.
2	200	50	0	50	0	0
3	500	100	50	200	50	50
4	500	100	50	500	100	50
5	100	50	50	200	50	0
7	100	50	50	200	0	0
10	50	0	0	100	0	0

*From glucose asparagine medium containing 1% glucose, 0·05% each of asparagine and dipotassium phosphate.

Summary

Xanthomonas malvacearum (E.F.Sm.) Dowson and *Erwinia carotovora* (Jones) Holland, when added to sterile soil as cell suspensions, survived for 153 and 171 days respectively. When added along with the infected host tissues, their survival period was 62 and 79 days. In unsterilized soil they survived for 110 and 121 days respectively, when added as cell suspensions, and for 24 and 31 days when added along with the host tissues. The survival for a shorter period of the pathogenic bacteria in the infected host tissues in soil was probably due to the antagonistic actinomycetes colonizing the tissues.

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TAXONOMIC STUDY OF *CRYPTOCOCCUS* SPP. ASSOCIATED WITH MAN

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AND
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(Received for publication, May 1961)

The genus *Cryptococcus* Kützing *emend.* Vuillemin (Lodder and Kreger-van Rij, 1951) comprises of asporogenous, capsulated and nonfermenting yeasts capable of synthesizing starch. No pseudo or true mycelium is produced. Lodder and Kreger-van Rij (1951) have described 5 species and 3 varieties. Since then 3 new species, namely, *C. terreus* (di Menna, 1954), *C. gastricus* (Reiersöl and di Menna, 1958) and *C. nigricans* (Rich and Stern, 1958), have been reported. *C. neoformans* is the only species of the genus known to cause disease in man and animals (Conant, Smith, Baker, Callaway and Martin, 1954; Ainsworth and Austwick, 1959). From India Khan, Ruth Myers and Koshy (1959) have reported *C. neoformans* from the sputum of a human case of pulmonary cryptococcosis. More recently Verma, Sriramachari, Pillai, Sridhara Rama Rao, Ramananda Rao and Sirsi (1960) reported *C. neoformans* from cerebrospinal fluid of a patient.

In the course of investigations on the role of fungi in pulmonary diseases and in skin infections of man, the authors have isolated *Cryptococcus* spp. A detailed taxonomic study of these is reported in this paper.

Materials and Methods

Clinical specimens like fresh morning sputa, throat swabs, bronchial aspirates, etc., were collected under aseptic conditions from the patients mostly with non-tuberculous pulmonary diseases attending the Research Clinic of the Institute. Some cases of skin diseases were also investigated by taking skin scrapings from the infected parts of the body. The specimens were cultured on the following media (Beneke, 1957): (1) Sabouraud agar (peptone 1 g.; glucose 4g.; agar 2g./100 ml. of distilled water; *p*H 5·6), (2) Sabouraud agar with penicillin 20 units and streptomycin 40 units/ml. of the medium, (3) Sabouraud agar with penicillin and streptomycin as above plus 0·5 mg. of actidione (cycloheximide)/ml. of the medium and (4) brain-heart infusion blood agar (brain-heart infusion 3·7 g.; blood 6 ml.; agar 2g./100 ml. of distilled water). Penicillin and streptomycin were also incorporated into this medium. The cultures were incubated at 25°C for 3 to 4 weeks for the appearance of pathogenic fungi.

In primary cultures, yeast colonies appeared in 2 to 3 days. They were subcultured on malt agar slants and maintained at 25°C for further study. The following criteria have been used for identifying the genus *Cryptococcus*: (1) presence of capsule around the cell, as observed in India-ink preparation, (2) absence of pseudo- or true mycelium, (3) inability to ferment sugars, (4) synthesis of starch and (5) positive urease test (Seelinger, 1956). Species identification was based on assimilation pattern of the isolates, using potassium nitrate as sole source of nitrogen and glucose, galactose, sucrose, maltose and lactose as sole sources of carbon.

In the various tests mentioned below, 2 to 3 days old streak cultures on malt agar slants at 25°C were used as source of inoculum.

Of the various media recommended (Lodder and Kreger-van Rij, 1951) for ascospore formation only Gorodkowa agar, potato and carrot plugs were tried. Inoculation was done by smearing the culture on the surface of plugs and agar medium. After 2 and 4 weeks of incubation at 25°C, smear preparations were made on glass slides, fixed by heat and stained with 5% aqueous solution of malachite green at 80°C for 3 to 5 min. The slides were washed in running water for 30 sec. and the cells were counterstained with ·5% aqueous safranin for 10 sec. The ascospores are stained blue and the vegetative cells red.

Dalmau cultures (Wickerham, 1951) were made for observing the development of true or pseudomycelium. In a Petri-dish containing potato-dextrose-agar, one streak and two point inoculations were made with a light suspension of yeast cells. Two sterile cover glasses were then placed, one across the streak and the second centering over one of the point inoculations. After 3 to 5 days incubation at 25°C growth was observed by taking off the lid and examining the Petri-dish directly under the microscope.

Growth characters like ring, pellicle and sediment formation were observed in malt extract broth. One hundred ml. Erlenmyer flasks, each containing 30 ml. of the broth (3% dehydrated malt extract), were inoculated with a drop of cell suspension and incubated in duplicate at 25°C for 3 days and at 17°C for one month. The size and shape of the cells were noted in 3 days old cultures at 25°C.

Gross and microscopic morphology was studied in streak cultures on malt agar slants incubated in duplicate at 25°C for 3 days and at 17°C for one month.

Presence of capsule was observed in germicidal India-ink preparations (India-ink 15 ml.; merthiolate 1/1000 solution 30 ml.; Tween 80 1/100 solution 0·1 ml.).

Fermentation tests were done in Durham tubes containing 2% solution of various sugars in 1% yeast extract with bromothymol blue as indicator. A set of five tubes, containing glucose, galactose, sucrose, maltose and lactose and one control set without any sugar, were inoculated with yeast cells and incubated at 25°C. Observations on gas formation were made periodically up to 3 weeks.

Sugar assimilation tests were done in liquid medium. Tubes containing 5 ml. of a synthetic mineral and vitamin solution (Lodder and Kreger-van Rij, 1951), containing the five sugars used for fermentation tests, were inoculated with yeast cells and incubated at 25°C. Readings on growth were taken after one, two and three weeks according to the method of Wickerham (1951) and 2+ and 3+ categories were taken as positive growth while 1+ as weak growth.

Formation of starch was tested in sugar assimilation tubes, at the end of 3 weeks, by adding a drop of 0·02N iodine solution. Appearance of blue colouration indicated positive result.

A tube containing Wickerham's (1951) nitrate assimilation medium was inoculated with a drop of light yeast cell suspension and incubated for a week at 25°C. From this, a subculture was made and incubated for a week. Observations on growth were made in the same way as for sugar assimilation tests.

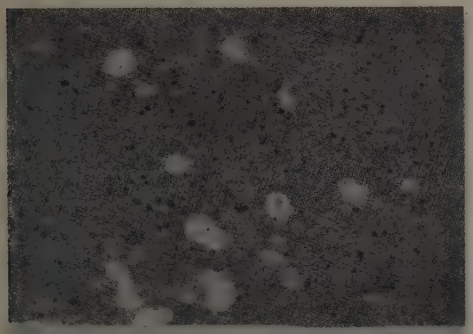
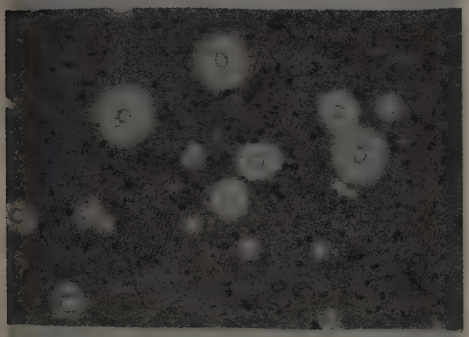


Fig. 1. *Cryptococcus diffluens* showing capsules; India-ink preparation 500 X.



Fig, 2. *C. laurentii* showing capsules; India-ink preparation 500 X.

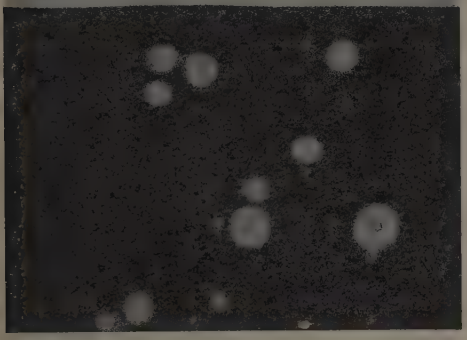


Fig. 3. *C. albidus* showing capsules; India-ink preparation 500 X.

The ability of various isolates to grow on ethanol as sole source of carbon and to split arbutin was tested according to the methods of Lodder and Kreger-van Rij (1951).

Urease test, as recommended by Seelinger (1956), was performed. Slants of Christensen's medium were thickly seeded with the isolates and incubated at 25°C.

Urea hydrolysis was indicated by a distinct colour change from orange yellow to a deep pinkish red.

Results

Of the seven isolates studied in detail, five belonged to *C. diffluens* and one each to *C. albidus* and *C. laurentii*.

C. diffluens (Zach) Lodder and Kreger-van Rij.

Four strains were isolated from the sputa of cases of respiratory diseases and one from skin scrapings of a case of skin infection.

The cells, after 3 days growth at 25°C in malt extract, were mostly round, 2·7—6·8μ, short oval or oval, 2·7—4μ×3·5—6μ, single, in pairs or occasionally in small groups (Fig. 4A; Fig. 1). A sediment was present but no ring or pellicle was formed. After one month at 17°C a heavy, yellowish and slimy sediment was formed while ring and pellicle were absent.

The cells were round to oval 2·5—3·5μ×3·5—5μ, occasionally up to 6·5μ after 3 days growth on malt agar at 25°C. After one month at 17°C, streak culture was cream to yellowish in shade, wet, shiny and mucoid. Surface and margin were usually smooth. Occasionally a few minute depressions were present on the surface and the margin was lobed. There was no growth at 37°C.

No true or pseudomycelium was produced in Dalmau cultures.

Fermentation was absent; excepting lactose all the sugars tested, were assimilated; glucose, sucrose and maltose assimilation tubes gave positive test for starch; assimilation of potassium nitrate was positive and well developed creeping pellicle was usually present; no growth or very weak growth on ethanol after one month; and splitting of arbutin and urease tests were positive.

C. albidus (Saito) Skinner

The strain was isolated from sputum of a case of respiratory disease.

The cells, after 3 days growth at 25°C in malt extract, were round, 2·8—6·5μ in diameter, short oval or oval, 2·5—4μ×4—5·5μ, single, in pairs (Fig. 4B; Fig. 2) or occasionally in groups of 3 to 4. Pellicle was absent and ring was rudimentary. Some bottom growth was present. After one month at 17°C there was a heavy yellowish sediment and poorly developed ring. The medium was turbid due to suspended growth. The cells showed medium to large sized capsules.

The cells were round 2·5—5·5μ in diameter, or short oval measuring 2·5—5μ×3·5—5·5μ after 3 days growth at 25°C on malt agar. After a month at 17°C the streak culture was cream to pale buff in colour, mucoid and wet. Surface was smooth with a few wrinkles at some places and margin was wavy. There was no growth at 37°C upto 3 days.

No true or pseudomycelium was formed in Dalmau culture.

Fermentation was absent; all the five sugars were assimilated; glucose and sucrose assimilation tubes gave positive test for starch; assimilation of potassium nitrate was positive; very weak growth on ethanol; and splitting of arbutin and urease tests were positive.

C. laurentii (Kufferath) Skinner

The strain was isolated from the throat of a patient.

The cells, after 3 days growth at 25°C in malt extract, were oval or round, single, in pairs, or sometimes in small groups. The round cells measured 2·5—5·5μ and the oval cells measured 2·5—4μ×4—6μ (Fig. 4C; Fig. 3). Sediment was present but no ring or pellicle was formed. After a month at 17°C a well developed creeping ring and a pellicle were formed. The pellicle was slimy and indistinguishable from the medium in colour. The entire contents of the flask changed into a somewhat slimy mass. Abundant yellowish sediment was formed. The cells were surrounded by a capsule.

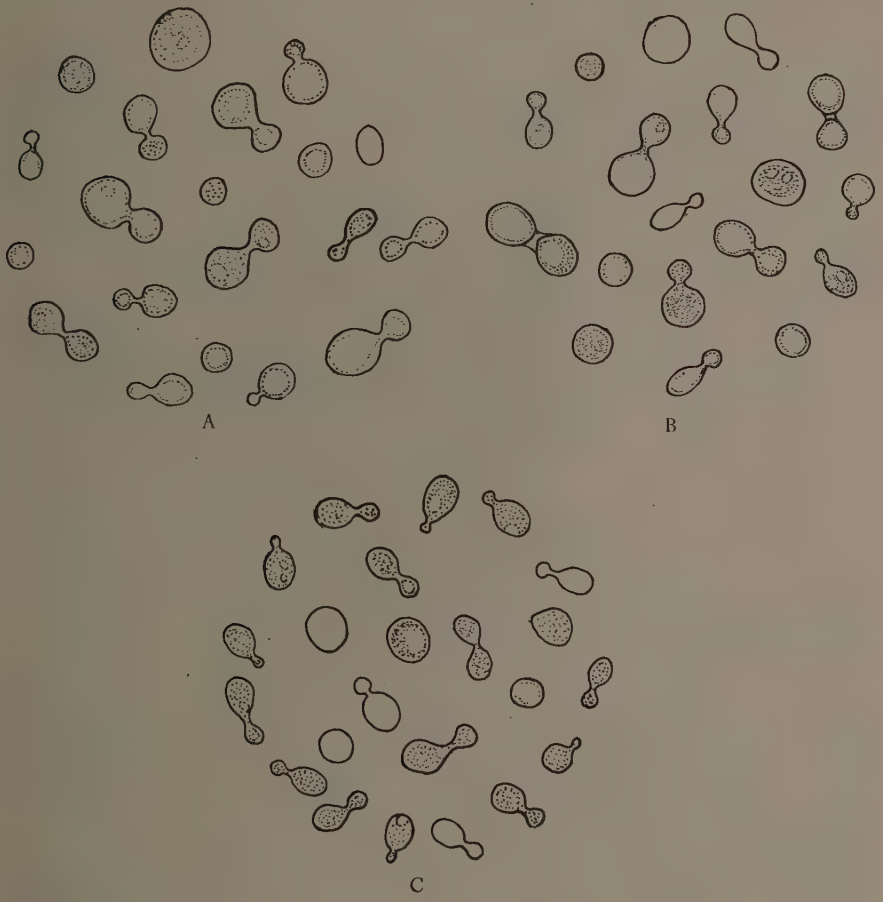


Fig. 4. Species of *Cryptococcus* (three days old growth in malt extract at 25°C); camera lucida drawings 1900 X.

The cells were short oval to oval, 2·5—4μ×3—6·5μ after 3 days growth at 25°C on malt agar. Some cells were round and very small, about 2μ in diameter. After a month at 17°C the streak culture was pale yellow to pale brown, mucoid, glistening and wet. Surface and margin were smooth. There was good growth at 37°C.

No true or pseudomycelium was formed in Dalmau cultures.

Fermentation was absent; excepting lactose all the sugars tested, were assimilated; starch was synthesized in all the assimilation tubes; assimilation of potassium nitrate was negative; no growth on ethanol; splitting of arbutin weakly positive and urease test was positive.

Since this strain was able to grow well at 37°C, it was considered desirable to test its pathogenicity in white mice. Intravenous injections were given to 3 male mice each receiving 22, 44, and 66 million cells respectively. The organism could be recovered in culture upto 4 weeks but they did not infect the animals.

Discussion

The three species described above showed a broad agreement with the descriptions given by Lodder and Kreger-van Rij (1951). Some minor variations in cell size, etc., however, occurred. The finding, of a consistently positive urease test in all the isolates, confirms the usefulness of this test in identification of the genus *Cryptococcus*, as advocated by Seelinger (1956). No aetiologic role could be ascribed to any of the isolates because positive cultures were obtained only on one occasion and could not be confirmed on repetition and the number of colonies appearing in primary cultures were few. One isolate of *C. laurentii* that grew at 37°C proved non-pathogenic for mice.

Summary

A detailed description of the morphological and physiological characteristics of seven isolates of *Cryptococcus*, five representing *C. diffluens* and one each representing *C. albidus* and *C. laurentii* has been given. The isolates have been obtained from clinical specimens of patients.

Acknowledgment

The writers are thankful to Dr. Libero Ajello, Communicable Disease Center, Atlanta, U.S.A. for checking one strain of *C. diffluens*, to Dr. I. M. Gupta for histopathological investigations of tissues of the experimental animals, to Mr. V. Ramanathan for photomicrographs, to Mr. J. N. Sharma for his technical assistance and to Dr. R. Viswanathan, Director, for his encouragement in this work.

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COMPARATIVE STUDY OF PARENT AND THE VARIENTS OF *HELMINTHOSPORIUM SATIVUM* OBTAINED BY ULTRA VIOLET IRRADIATION

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Helminthosporium sativum P. K. and B., the organism causing leafspot of corn or maize (*Zea mays* L.) is known to show variability in nature and in culture media. Greaney and Machacek (1933) induced saltations in this fungus by exposing it to ultra violet light. According to Christensen and Davies (1937) new races of *H. sativum* frequently arise as sectors in cultures on artificial media. Such variabilities in this genus have been attributed to heterokaryotic conditions of the asexual mycelium and mutation (Dickinson, 1932; Shands and Dickson, 1934; Graham, 1935; Robert, 1952; Tinline, Stanffer and Dickson, 1960; Chattopadhyay and Dickson (1960).

The effect of U. V. irradiation on a strain of *H. sativum*, isolated locally from the leafspot of maize is presented in this communication.

Materials and Methods

Irradiation of the fungus : In a preliminary study the mycelial colonies of the fungus, grown on potato dextrose agar in Petri-dishes, were exposed to U.V. light, but the results were not satisfactory. Shorter exposures did not cause any apparent change in

the fungus and longer exposures resulted in the heating up and subsequent drying of the of the agar medium. The method described by Chattopadhyay and Dickson (1960), in which a suspension of germinating conidia and hyphae was exposed to the U.V. light, was then tried and found to be satisfactory. Therefore, this method was mainly used to obtain mutants. Hanovia Prescription Lamp, Model-4 was used as the source of light. The distance between the source of light and the Petri-dish was 10 or 15 cm. The time of exposure to the light varied from 12 to 18 min. Suitable aliquots of germinating conidia and hyphae, suspended in sterile distilled water in Petri-dishes, were irradiated with the U.V. light, with the lid of the dishes removed. Soon after the treatment the lids were replaced. Melted potato dextrose agar (P.D.A.) was poured into the dish, the contents mixed thoroughly to get a uniform distribution of the conidia and incubated at room temperature (26° to 28°C) for 3 to 7 days. The fungal colonies developing in the medium were examined and those showing any variation in pigment colour or in morphological characters were subcultured.

Cultural studies: The details regarding the procedure used for purifying the fungus, preparation of the media, culturing etc., are the same as described in the Manual of Microbiological Methods (Anonymous, 1957). To compare the relative growth of the isolates on solid media, 4 mm. discs of the fungus were cut with a sterile cork borer from a fast growing region of a colony and inoculated on agar media in the centre of the Petri-dishes. The radial spread of the mycelium was measured at periodic intervals. To compare the growth in liquid media, 75 ml. of liquid broth was put in each of the 250 ml. Erlenmeyer flasks and inoculated with fungus as described above. On the 13th day the dry weight of the fungal mat was determined in the usual manner. In all cases quardruplicates were maintained and the average weight was calculated.

Infectivity studies: The infectivity of the fungus was tested by transferring mycelial mat, taken from a 7 day old culture, to the healthy leaf surface, previously sterilized in the usual manner with mercuric chloride and washed with sterile water. The inoculum was then covered with a piece of sterile wet cotton wool. The plants were covered with alkathene cases and high humidity maintained inside by frequent sprayings with water. Periodic observations on the development of disease symptoms were made. Wherever the inoculation was successful, the pathogen was reisolated from the infected area and compared with the original isolate.

To test the infectivity of the fungus on the seeds of wheat and oat, the seeds were first surface sterilized in the usual manner with mercuric chloride, inoculated by soaking in a suspension of the fungal conidia for 6 to 8 hr. and sown on moist filter paper in Petri-dishes. Seeds were also kept as controls. Observations on the percentage of germination and pre- and post-emergence rot of the seeds were made. Fungus from the infected seeds and seedlings was isolated and compared with the original strain.

Results

In most cases U.V. radiation was lethal and the germ tubes and hyphae failed to develop into colonies. Whenever colonies developed, they were examined carefully. In all 54 variants were obtained on P.D.A. slants. Two of them (M-1 and M-2), which showed distinct difference in characters from the parent strain and maintained these characters even after repeated subculturing, were selected for detailed investigations.

A comparison of the morphological characters of the parent strain and the mutants is given in Table I. The parent and M-1 mutant were found to resemble in their hyphal characters, but the hyphae of the latter were more narrowly septate. In M-2 the condial heads were dwarfed and only rarely conidia were formed. Such conidia appeared normal but no measurements were taken.

TABLE I

A comparison of the morphological characters of the parent and mutant strains of H. sativum

	Parent	Mutant-1	Mutant-2
Mycelium and pigmentation	Compact, velvetty and black olivaceous aerial growth	Compact, velvetty light brown aerial growth	Compact greyish white profuse aerial growth
Hyphae	Septate, subhyaline	Septate, subhyaline but septation narrower than in parent	Dwarfed, characteristic conidial heads
Sporulation and conidia	Septate, narrower at the middle and dark olivaceous brown	Septate, broader than in parent isolate, lighter coloured, more sporulation than in the parent isolate	Conidia normal but very few

There was considerable difference in the spore size of the M-1 mutant; it was significantly shorter and broader than the conidia of the parent strain (Table II).

TABLE II

Comparison of the spore sizes of the parent and M-1 mutant strain of H. sativum

(100 spores were measured in each case)

Particulars	Conidial size (μ)	Mean (μ)	Standard error for the difference of mean	't'	Degrees of freedom	Remarks
LENGTH OF CONIDIA						
Parent strain	39—111	74·01	10·61	—	—	
M-1 mutant	30—87	55·56	12·62	10.34	198	Highly significant
BREADTH OF CONIDIA						
Parent strain	4—18	12·23	1·064	—	—	
M-1 mutant	9—21	13·95	3·729	3·256	198	Highly significant

The parent and mutant strains grew in all the media tested, M-2 was relatively slow grower (Table III).

TABLE III

Comparison of the growth of parent strain and M-1 and M-2 mutant of H. sativum

Medium	Growth in agar medium in mm.*			Growth in liquid medium in mg./75 ml.**		
	Parent	M-1	M-2	Parent	M-1	M-2
Potato dextrose	81	79	47	647	556	462
Oat meal	77	64	47	466	496	439
Yeast extract dextrose	80	77	60	513	528	461
Richard's	67	57	45	516	485	406
Czapek's	84	84	57	558	636	485

*Readings of radial growth of fungal colony taken on 7th day.
**Readings of dry weight of the mycelial mat taken on the 13th day.

The relative capacity of the strains to utilize carbon sources was tested in Czapek's medium. It was found that the isolate M-1 could utilize starch and fructose better than the parent strain and M-2 grew very slowly.

The infectivity of the parent and mutant strains on maize leaves is given in Table IV. All the strains infected leaves causing spots, M-1 caused higher percentage of infection compared with the parent strain, 98 and 91% respectively. Strain M-2 infected only 33% of the leaves.

TABLE IV

Infectivity of the parent and mutant strains of H. sativum *on maize leaves*

Isolate	Total number of leaves inoculated	Number of leaves infected					Infection (%)
		Number of days after inoculation					
		2	3	4	5	10	
Parent strain	120	53	90	102	106	109	91
M-1 mutant	108	100	104	105	106	106	98
M-2 mutant	140	13	24	29	41	46	33

Since *H. sativum* is known to cause pre- and post-emergence rot of some cereals, healthy wheat and oat seeds were treated with the conidial suspensions of the parent and mutant M-1 strains, in separate lots. The seeds, loaded with the conidial mass, were sown in sterilized soil in pots as well as on moist filter papers in Petri-dishes. The percentage of germination and the percentage infection of the seeds are presented in

Table V. In the case of parent strain, though there was normal seed germination, the seedling infections were apparent when they were 2 to 3 mm. in height. The seed germination was much delayed in the case of M-1 mutant, especially in the case of oats, and there was severe stunting of the seedlings. The control seeds germinated quicker and there was faster growth of the seedlings.

TABLE V

Infectivity of the parent and M-1 mutant of H. sativum *on wheat and oat seedlings*

Particulars	Total number of seeds sown	Number of seeds germinated on				% germination	Number of seeds infected on				Infection* (%)
		3rd day	4th day	5th day	7th day		3rd day	4th day	5th day	7th day	
WHEAT											
Control	40	33	33	35	36	90	—	—	—	—	—
Parent strain	40	20	23	27	27	67	3	8	11	16	44
M-1 mutant	40	26	26	28	29	72	7	11	14	21	58
OAT											
Control	24	8	10	11	14	58	—	—	—	—	—
Parent strain	24	5	7	9	10	62	—	2	4	5	36
M-1 mutant	24	6	8	11	13	54	1	4	6	9	64

* Percentage of infection was calculated on the basis of the total number of seeds germinating in the control.

Discussion

The mutants of *H. sativum* differed from the parent strain in some morphological characters. The dwarfed, stunted and distorted hyphae of mutant M-2 appears to be similar to those reported by other workers. Greaney and Machacek (1933) and Dierner (1955) observed distortion or formation of protruberances, stunting, suppressed aerial growth and dwarfed conidial beads in the mutants. In the case of M-2 there was also a distinct reduction in the growth rate similar to that reported by Greaney and Machacek (1933), Hollaender, Kenneth and Coghill (1945) and Dierner (1955). The change in chromogenesis was observed in both the mutants; in M-1 the pigment was lighter and in M-2 it was darker than the parent strain. Similar changes in the mutants of other fungi have been reported by Nandi and Mishra (1958) and Tinline, Stanffer and Dickson (1960). Due to U.V. irradiation sporulating capacity of M-2 was lost while it increased in M-1 as compared with the parent strain. Ramsey and Bailey (1930) and Dierner (1955) have obtained cultures with increased sporulating capacity due to mutation by irradiation and Dierner (1955) and Sahaguchi, Suzuki and Iizuka (1955) have obtained non-sporulating mutants.

Summary

By irradiating a suspension of the germinating conidia and hyphae of *Helminthosporium sativum* P.K. and B. to U.V. light, two mutants were obtained. One of them was characterized by lighter coloured mycelium, faster growth in agar and liquid media, more conidial production, increased percentage of infectivity of the leaves of maize and causing pre- and post-emergence rot of wheat and oat seedlings; the other was characterized by darker coloured mycelium with stunted, dwarfed and narrowly septate hyphae and reduced conidial heads, loss of sporulating capacity, much reduced growth rate in liquid and agar media and reduced percentage of infectivity as compared with the parent strain.

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STUDIES ON THE GROWTH OF *MYCOBACTERIUM TUBERCULOSIS* STRAIN H37Rv IN ISAO YAMANE'S MEDIUM AND ITS COMPARISON WITH OTHER ROUTINELY USED MEDIA

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It is well known that *Mycobacterium tuberculosis* grows slowly in culture and it is difficult to initiate its growth from very small inoculum. A number of successfully used routine media for *M. tuberculosis* require egg as an essential constituent. Yamane (1957) described the isolation of a crystalline substance from egg-yolk which promoted the growth of minute inoculum of human tubercle bacilli in solid medium. Boissevain and Schultz (1938) and Dubos and Davis (1946) have already described the growth promoting activity of this substance for *M. tuberculosis*. The latter workers have also given the disadvantages of the use of egg-yolk isolate. In view of these findings, it was considered worthwhile to investigate further Yamane's medium for growing *M. tuberculosis* and the the results are presented in this communication.

Materials and Methods

M. tuberculosis (strain H37Rv) was very kindly supplied by Prof. Dr. R. L. Mayer, Director of Microbiological Research, CIBA Pharmaceutical Products Inc., Summit, N. J. The stock culture was maintained on Loewenstein Jensen medium.

The egg-yolk isolate was extracted from white Leghorn and Rhode Island eggs by the method described by Yamane (1957).

The liquid media used (Table I) were Yamane (1957), Youmans with horse serum (Youmans and Karlson, 1947), Youmans with coconut water (Ramkrishnan, Indira and Sirsi, 1958) and modified Kirchner (Eisman, Konopka and Mayer, 1954).

TABLE I

Components of the media	Yamane	Youmans with horse serum	Youmans with coconut water	Modified Kirchner
$Na_2HPO_4.12\ H_2O$	6·0 g.	—	—	3·0 g. of $Na_2HPO_4.7H_2O$
KH_2PO_4	3·0 g.	5·0 g.	5·0 g.	4·0 g.
Monosodium glutamate	5·0 g.	—	—	—
Ferric ammonium citrate	0·1 g.	—	—	0·05 g.
Asparagine	0·5 g.	5·0 g.	5·0 g.	5·0 g.
$(NH_4)_2SO_4$	0·5 g.	—	—	—
Calcium pantothenate	0·1 g.	—	—	—
Sodium citrate	—	—	—	2·5 g.
Magnesium citrate	—	1·5 g.	1·5 g.	—
Magnesium sulphate	—	—	—	0·6 g.
Potassium sulphate	—	0·5 g.	0·5 g.	—
Malachite green (2% solution)	0·7 ml.	—	—	—
Glycerol	—	20·0 ml.	20·0 ml.	20·0 ml.
Tween—80	5·0 g.	—	—	0·3 ml.
Growth promoter	0·1 g. crystalline egg-yolk isolate	100 ml. horse serum	100 ml. tender coconut water	5·0 g. serum albumin
Distilled water (all-glass distilled)	to make 1 litre	to make 1 litre	to make 1 litre	to make 1 litre

Yamane's solid medium was prepared by the addition of 2·5% defatted purified agar to the liquid medium given in Table I.

Borosilicate test tubes (20×150 mm.) were used for growing *M. tuberculosis* in liquid media. The final liquid volume in each tube was always adjusted to 5 ml. in the experiments described below. The tubes were plugged with absorbent cotton, as suggested by Dubos and Davis (1946).

Results

Comparison of growth of M. tuberculosis *in Yamane and Loewenstein Jensen solid media*

Using heavy inoculum

From a 7 days old culture of *M. tuberculosis* on Loewenstein Jensen slant, a loopful of the bacteria was inoculated on Yamane's slant and another loopful on Loewenstein

Jensen and incubated at 37°C. Visible growth appeared on Yamane in 3-4 days and in 6-7 days the entire slant was covered with colonies having raised dense centre and wavy surface. On Loewenstein Jensen, visible growth appeared on the 6th day and it was maximum by the 14th day.

Using small inoculum

A 7 days old culture of *M. tuberculosis* on Loewenstein Jensen slant was harvested and suspended in normal saline, giving an opacity equal to Brown's barium sulphate opacity tube No. 4. From this original suspension further dilutions were made (10^{-1}, 10^{-2}, 10^{-3} and 10^{-4}). The original suspension and each of the dilutions was inoculated into 3 tubes of Yamane and 3 of Loewenstein Jensen, each tube receiving an inoculum of 0·05 ml. On Yamane's medium only slight growth was seen on the 9th day from the inoculum of the original suspension and no growth from any one of the dilutions. One colony could be seen on the 14th day and 5 colonies after 24 days. In Loewenstein Jensen medium, the original suspension as well as 10^{-1} and 10^{-2} dilutions gave very thick confluent growth in 14 days. An average of 56 and 5 colonies per tube was obtained from 10^{-3} and 10^{-4} dilutions respectively.

Comparison of growth of M. tuberculosis *in different liquid media*

Using heavy inoculum

One loopful of *M. tuberculosis* from a 7 day's old culture on Loewenstein Jensen slant was inoculated into each tube containing different liquid media and the appearance of the growth was studied up to 14 days (Table II).

TABLE II

Growth of M. tuberculosis *in liquid media using heavy inoculum*

Liquid media	Growth at intervals in days										
	1	2	3	4	5	6	7	8	10	12	14
Yamane (without agar)	—	—	±	+	++	++	+++	+++	++++	++++	++++
Youmans with 10% horse serum	—	—	—	—	—	+	+	++	+++	+++	++++
Youmans with tender coconut water	—	—	—	—	—	+	+	++	+++	+++	++++
Modified Kirchner	—	—	—	—	—	+	+	++	+++	+++	++++

— =No growth; ± =doubtful growth; +, ++, +++ and ++++ show increasing amount of growth.

Using small inoculum

A 7 day's old culture of *M. tuberculosis* on Loewenstein Jensen slant was harvested and suspended in normal saline to give an opacity equivalent to Brown's standard barium sulphate opacity tube No. 4 (approximately 10^6 bacilli/ml.). 0·1 ml. of this suspension was inoculated into each medium and incubated at 37°C. The appearance of growth was observed up to 14 days (Table III).

TABLE III

Growth of M. tuberculosis *in liquid media using small inoculum*

Liquid media	Growth at intervals in days										
	1	2	3	4	5	6	7	8	10	12	14
Yamane (without agar)	—	—	—	—	—	—	—	—	+	++	++
Youmans with 10% horse serum	—	—	—	—	—	—	—	—	+	+++	++++
Youmans with tender coconut water	—	—	—	—	—	—	—	—	+	+++	++++
Modified Kirchner	—	—	—	—	—	—	—	—	+	++	++++

— – No growth; +, ++, +++ and ++++ show increasing amount of growth.

Inhibition of growth of M. tuberculosis *by PAS and INH in different liquid media*

A suspension of *M. tuberculosis* in normal saline was made as already described above (containing approximately 10^6 cells/ml.) and 0·1 ml. of this was inoculated in each of the medium containing different concentrations of PAS and INH. The tubes were incubated at 37°C for 21 days. The results are presented in Table IV.

Effect of replacing asparagine by tryptone or glutamate from Yamane's liquid medium on the growth of M. tuberculosis

Liquid medium based on Yamane was prepared without asparagine and divided into three equal parts. In the first was added asparagine (0·5 g./1000 ml. of the medium, as suggested by Yamane), in the second the same amount of tryptone was added in place of asparagine and in the third monosodium glutamate (in addition to the glutamate already present in the medium). All the three media were then inoculated with a loopful of H37Rv from a 7 days old culture on Loewenstein Jensen medium. The growth observed during the period of 21 days is shown in Table V.

Effect of replacing Tween-80 by glycerine from Yamane's liquid medium on the growth of M. tuberculosis

Yamane medium prepared without Tween-80 was divided equally into 3 parts. In one was added Tween-80 (0·5% concentration), in the second glycerine (2·0% concentration) and the third kept as control. Each of the medium was inoculated with a heavy inoculum of H37Rv and incubated at 37°C. The growth of bacteria during 21 days is shown in Table VI.

TABLE IV

Concentrations of PAS and INH inhibiting the growth of M. tuberculosis *in the different liquid media*

Liquid media	Concentrations of PAS in μg./ml.							Concentrations of INH in μg./ml.						
	1·0	0·8	0·6	0·5	0·4	0·2	0·1	0·1	0·08	0·06	0·04	0·03	0·02	0·01
Yamane	—	—	—	+	+	++	++	—	—	—	+	+	++	++
Youmans with 10% horse serum	—	—	—	++	+++	++++	++++	—	—	—	+	++	++	+++
Modified Kirchner	—	—	+	++	+++	++++	++++	—	—	—	+	++	++	+++

— = No growth; +, ++, +++ and ++++ show increasing amount of growth.

TABLE V

Effect of replacing asparagine by tryptone or glutamate from Yamane's liquid medium on the growth of M. tuberculosis

Liquid media	Growth at intervals in days								
	1	2	3	4	5	6	7	8	21
Yamane	—	—	±	+	++	++	+++	+++	++++
Yamane with tryptone in place of asparagine	—	—	—	+	+	+	++	++	++
Yamane with glutamate in place of asparagine	—	—	—	—	—	—	±	+	+

— = No growth; ± = Doubtful growth; +, ++, +++ and ++++ show increasing amount of growth.

TABLE VI

Effect of replacing Tween-80 by glycerine from Yamane's liquid medium on the growth of M. tuberculosis

Liquid media	Growth at intervals in days								
	1	2	3	4	5	6	7	8	21
Yamane	—	—	±	+	++	++	+++	++++	++++
Yamane with glycerine in place of Tween-80	—	—	—	—	+	+	++	++	++
Yamane without Tween-80	—	—	—	—	±	+	+	+	+

—=No growth; ± Doubtful growth; +, ++, +++ and ++++ show increasing amount of growth.

Virulence of M. tuberculosis *maintained on Yamane's solid medium*

H37Rv strain maintained on Yamane's solid medium, by sub-culturing for over one year, was tested for its virulence.

Cytochemical test

Washed bacterial cells were suspended in 5% aqueous solution of NaCl containing 1% sodium barbiturate and then treated with a dilute solution of neutral red for Dubos and Middlebrook (1948) test. The bacterial cells became bright red, giving positive test for virulence.

Mice infectivity

Six Haffkine Institute inbred mice (average weight 27 g.) were intraperitoneally injected with 0·5 ml./mice of a 7-day's old culture in Yamane's liquid medium. After 2 months the mice were sacrificed. The lungs, spleen and liver of all the mice showed extensive tubercles. Smears of these organs showed the presence of typical acid-fast bacilli. Positive cultures were obtained on Loewenstein Jensen medium from the homogenates of the organs.

Guinea-pig infectivity

Guinea-pigs weighing 350-400 g. were intraperitoneally injected with a 7 day's old culture of H37Rv in Yamane's liquid medium (2·5 ml./animal). All the animals died in 3-4 months. Post mortem examination revealed typical progressive tuberculosis. Lungs were studied with tubercles and spleen was highly enlarged and full of tubercles. Omentum was thickened, rolled and converted into a solid mass. Microscopic examinations and cultural tests confirmed the presence of tubercle bacilli in the affected organs.

Discussion

Yamane (1957) proposed a medium, containing the crystalline egg-yolk isolate, for the primary isolation and plate count of *M. tuberculosis*. Our results have shown

that this medium gives rapid growth of bacteria only when a heavy inoculum is used. With diluted inocula there is no growth or very poor growth of bacteria, even on longer incubation. Therefore, this medium has limitations for primary isolation or plate count. Loewenstein Jensen medium stands superior, in this regard, as it gave the number of colonies proportional to the concentration of the inoculum used. The unsatisfactory performance of media containing Tween-80 or other wetting-out agents, and the superiority of Lowenstein Jensen medium among the routinely used media, has been stressed recently by Coletsos (1960).

The comparison of the different liquid media has confirmed the finding of Ramkrishnan, Indira and Sirsi (1958) regarding the growth-promoting activity of tender coconut water, when added to Youman's medium. The growth of *M. tuberculosis* in this medium was nearly equivalent to that obtained by the addition of 10% horse serum to Youman's medium. When a heavy inoculum of *M. tuberculosis* is given in the liquid medium of Yamane (1957), the growth of bacteria is much earlier than in any other liquid media studied and therefore, this can be used for the screening of potential antitubercular agents *in vitro*.

The importance of asparagine for the growth of tubercle bacilli in culture is already known. Some workers have advocated the use of glutamate or casein hydrolysate instead of asparagine. Yamane's medium requires 5·0 g. of glutamate and only 0·5 g. of asparagine per litre in comparison to 5·0 g. of asparagine per litre required by Youmans or modified Kirchners media. It has been shown in the present work that the presence of this small quantity of asparagine in Yamane's medium is essential. The replacement of asparagine by tryptone gave only partial growth and with glutamate still poorer growth resulted. Similarly, the importance of Tween-80 in Yamane's medium has been clearly brought out in our experiments wherein the elimination of Tween-80 or its replacement by glycerine gave very poor and delayed growth.

Strain H37Rv of *M. tuberculosis*, continuously sub-cultured on Yamane's solid medium for over one year, was found to maintain its virulence.

Summary

A medium proposed by Yamane for the growth of *Mycobacterium tuberculosis* has been studied and compared with other routinely used media. Claims made by Yamane for the growth of *M. tuberculosis* in his medium, when very small inoculum is used, could not be substantiated. Rapid growth was obtained, both in solid and the liquid medium of Yamane, when heavy inoculum was used. It is suggested that the liquid medium based on Yamane can be used, with heavy inoculum, for the screening of potential antitubercular compounds *in vitro*.

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SENSITIVITY OF LOCALLY ISOLATED STRAINS OF *STAPHYLOCOCCUS* AND OTHER PATHOGENIC BACTERIA TO PENICILLIN AND OTHER ANTIBIOTICS

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There is a possibility that in a closed area like a hospital ward, where various antibiotics are routinely employed, the bacterial flora may become resistant. Healthy individuals may carry in their nasopharynx coagulase positive staphylococci that are resistant to penicillin and other antibiotics and may serve as a source of infection to other individuals (Spink, 1961). Therefore a study was undertaken to find out the incidence of the resistant strains of *Staphylococcus aureus*, against penicillin and other antibiotics, isolated from the patients and the medical and nursing staff. Other bacterial strains isolated during routine examination were also tested against the antibiotics.

Material and Methods

One hundred and five strains of *S. aureus* were isolated from patients with pyogenic infections of the skin and subcutaneous tissues, attending the out-patient department of the King Edward VII Memorial Hospital from January to February 1953. Most of the material was collected when septic lesion was opened. No attempt was made to exclude patients who had received antibiotic treatment in the past. Fifty strains of *S. aureus* from nasal swabs were collected between October and December 1953 from in-patients suffering from various diseases who had stayed in the wards at least for one week. Between May and October 1954, fifty strains of *S. aureus* from 122 healthy members of the hospital staff, resident medical officers and the nursing staff were isolated from nasal swabs. *S. aureus* strains were also isolated from various bacterial infections like abscesses, furunculosis, whitlows, discharging sinuses, pleural fluid, wound infections, etc., in in-door patients, in the first half of 1960.

Other strains of pathogenic bacteria (*Bacterium coli*, *B. pyocyneus*, *S. haemolytics*, *S. nonhaemolyticus*, *Pneumoccus* sp., *Gonococcus* sp. and *Brucella melitensis*) were isolated

TABLE I

Antibiotic sensitivity of bacteria

Bacteria		Source	Total strains	Number of strains sensitive and resistant to									
				Penicillin		Chlorte-tracycline		Oxytetra-cycline		Strepto-mycin		Chloram-phenicol	
				S*	R+	S*	R+	S*	R+	S*	R+	S*	R+
Staphylococcus aureus	1953-54	Out-patients	105	98	7	105	0	105	0	—	—	—	—
	1954	In-patients	50	8	42	49	1	49	1	17	33	50	0
	1954	Staff	50	4	46	50	0	50	0	16	34	50	0
	1960	Staphylococcal infections	50	21	29	—	—	38	12	31	19	43	7
B. coli	1953-55	Patients attending K.E.M. hospital	10	—		4	6	4	6	9	1	10	0
	1960		43	0	43	—	—	16	27	26	17	25	18
B. pyocyaneus	1953		1	—	—	0	1	0	1	0	1	0	1
	1960		26	0	26	—	—	0	26	0	26	12	14
Streptococcus hemolyticus	1953-55		3	3	0	3	0	3	0	—	—	—	—
	1960		4	4	0	—	—	4	0	4	0	4	0
Streptococcus nonhemolyticus	1953-55		5	5	0	5	0	5	0	—	—	—	—
Pneumococcus sp.	1953-55		16	16	0	16	0	16	0	16	0	16	0
Gonococcus sp.	1953-55		1	1	0	1	0	1	0	—	—	—	—
Br. melitensis	1953-55		1	0	1	1	0	1	0	1	0	1	0
S. typhosa	1953-55	Other laboratories in Bombay	81	—	—	—	—	—	—	—	—	81	—
S. paratyphi A	1953-55		49	—	—	—	—	—	—	—	—	49	0

S* = sensitive; R+ = resistant

in the Department of Pathology and Bacteriology K.E.M. Hospital between 1954-55 and first half of 1960.

Strains of *Salmonella typhosa* and *S. paratyphi* A, isolated during 1950-53 were obtained from various bacteriological laboratories of Bombay.

The technique used for determining the sensitivity of micro-organisms to antibiotics was the agar diffusion method, using filterpaper disc (Gould and Bowie, 1952; Spaulding and Anderson, 1951). It was found to be reliable and simple.

Results

The sensitivity and resistance of strains of *S. aureus* and other bacteria towards different antibiotics are given in Table I. Out of the 105 strains of *S. aureus* isolated from the out-patients, only 7 were found to be resistant to penicillin and none against chlortetracycline and oxytetracycline (Table I). Pohujani (1960), working in the same institution tested 125 strains of *S. aureus*, collected from the out-patients, and found that 59 were resistant to penicillin. Forty two strains out of the 50, isolated from the nose of in-door patients, were penicillin-resistant. In the case of medical and nursing staff of the hospital, 46 strains of *S. aureus* out of 50 were sensitive to chlortetracycline, oxytetracycline and chloramphenicol. These results are in agreement with the findings of (Barber, Rozwadowska-Dowzenko, 1948; Barber, Hayboe, 1949; Birnstingl, Shooter, and Hunt, 1952; Finland and Haight, 1953).

There was a marked difference in the resistance of strains of *B. coli* and *B. pyocyaneus* towards different antibiotics. All strains of *S. typhosa* were sensitive to chloramphenicol (Table I).

Discussion

The appearance and dissemination of antibiotic-resistant strains of *S. aureus* are unique and no other bacterial species has posed such a major problem in clinical medicine. This species has become resistant not only against penicillin but also against several other important antibiotics. Vogelsang (1954) found the carrier-rate of pathogenic staphylococci in nasopharynx of 764 staff members of hospitals to be 76·4% and 47% of the strains isolated were resistant to penicillin. Martyn (1949) collected material from the nasopharynx of 130 new-born infants and found that 62% of these contained staphylococci. 55·5% of the strains isolated were resistant to penicillin. Bacteriophage typing of the strains has shown that only a small percentage of the infants carried the same strains as the mothers. Majority of the strains were similar to those found in the air of the nursery and in the nasal cavities of the nursing staff. Rountree and Thomson (1949), Rountree, Barbour, and Thomson (1951) and Rountree and Barbour (1951) have made important epidemiological observations on the establishment and spread of antibiotic-resistant strains of *Staphylococcus*. They found a close relationship between the incidence of resistant staphylococci and the use of antibiotics. The findings reported in the present work shows that 92% of the strains of *S. aureus*, isolated from the members of the hospital staff, and 84% from the indoor patients were resistant to penicillin, indicating the frequency of cross-infection. Gupta and Chakravarti (1954) from Lucknow, Myers and Acharya (1956) from Vellore, Pohujane

(1960) from Bombay, Sayed and Bhende (1958) from Ahmedabad and Jalihal and Sayed (1960) from Baroda have also reported the occurrence of strains of *S. aureus* resistant to penicillin and other antibiotics.

Summary

One hundred and five strains of *Staphylococcus aureus* isolated from the out-patients were tested for their sensitivity to penicillin, chlortetracycline and oxytetracycline. 7 strains were found resistant to penicillin but none to the other antibiotics. Out of 50 strains of *S.aureus*, recovered from the nasal swabs of hospital in-patients, 42 were found resistant to penicillin, 33 to streptomycin, 1 to chlortetracycline and oxytetracycline and none to chloramphenicol. Of the 50 strains of *S. aureus* recovered from nasal swabs of medical and nursing staff, 46 were found resistant to penicillin, 34 to streptomycin, and none to chlortetracycline, oxytetracycline and chloramphenicol. Sensitivity of other pathogenic bacterial strains to these antibiotics is also given.

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LETTER TO THE EDITOR

ANTIGENICITY OF CHEMICALLY PURIFIED BOTTLE GOURD MOSAIC VIRUS

A mosaic disease of bottle gourd (*Lagenaria siceraria* Standl.) has been described by Vasudeva, Raychaudhuri and Singh (1950), and Vasudeva, Raychaudhuri and Nariani (1954) and the virus designated as *Cucumis virus* 2C or *Marmor astrictum* var. *subobscurum* var. nov. Anand (1960) purified the virus by a chemical method. The present investigation was undertaken to test the antigenicity and purity of the virus.

A sample of the purified virus was mixed with an equal volume of 2N saline. A course of five 1 ml. intravenous injections of the antigen was administered to an albino rabbit, weighing about 1·8 kg., through its marginal vein at intervals of 7 days. The animal was then bled 15 days after the last injection. In all, four bleedings were done on weekly intervals. About 25 ml. of blood was collected from each bleeding. After clotting, the blood was centrifuged for 15 min. at 3,000 r.p.m. and a clear antiserum was obtained. All the four lots of the antiserum were pooled together and stored frozen at —10°C. in small narrow-mouthed glass-stoppered bottles.

The antiserum was tested against healthy and mosaic affected bottle-gourd plant saps. Two-fold dilutions of the antiserum and pure diseased plant sap were prepared using normal saline (0·85%). Diseased plant sap dilutions and antiserum dilutions (0·2 ml. each) were mixed in 7 mm. diameter serological glass tubes and incubated in a water-bath, maintained at 37°C. The time of appearance of flocculent precipitation was recorded.

The fastest precipitation was obtained in the tube containing 1 : 2 dilution of antiserum and 1 : 4 of the diseased plant sap, thereby giving the 'optimal proportion' or '$\propto$ Optimum' as : $\frac{\text{virus dilution}}{\text{antiserum dilution}} = \frac{1:4}{1:2} = \frac{1}{2}$. The '$\propto$ Optimum' was found to be the same for every horizontal row (Table I). The precipitation end point of the virus, with antiserum diluted to 1 : 8, 1 : 16, and 1 : 32, is 1 : 2048. No precipitation occurred at dilution 1 : 4096 after 24 hr. of incubation at 37°C. Similarly, no precipitation could be observed at the antiserum dilution of 1 : 128 after 24 hr. of incubation at 37°C. This could possibly be due to the fact that the antiserum was too diluted beyond 1 : 64 to form a visible precipitate. A negative result was obtained with the healthy plant sap. The normal serum obtained from an unimmunized rabbit also gave negative result with both the healthy and diseased plant saps.

TABLE I

Precipitation diagram of the antiserum, produced by five injections of 1 ml. each, of the purified bottle gourd mosaic virus in normal saline

Antiserum dilutions \ Virus dilutions	1:1	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024	1:2048	1:4096
1:1	2·30	1·30	1·45	2·35	5·00	10·00	24·00	50	60	+	+	—	—
1:2	1·00	0·30	0·25	0·35	2·10	6·30	16·00	47	48	+	+	—	—
1:4	2·00	1·55	0·45	0·35	1·50	6·00	13·00	42	47	+	+	—	—
1:8	6·00	5·30	2·10	2·00	1·50	5·30	12·30	40	45	+	+	—	—
1:16	40·00	30·00	21·00	12·00	9·55	8·30	12·00	35	40	+	+	+	—
1:32	—	122·00	115·00	49·00	39·00	35·00	30·00	46	50	+	+	+	—
1:64	—	—	—	—	+	+	+	+	+	+	+	—	—
1:128	—	—	—	—	—	—	—	—	—	—	—	—	—

+ =Precipitation observed after 24 hr. of incubation at 37° C.
— =No precipitation even after 24 hr. of incubation at 37° C.
(Figures denote timings in min. and sec.)

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